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FLASH INDUCED FLUORESCENCE KINETICS IN CHLOROPLASTS IN THE 20 μs–100 s TIME RANGE IN THE PRESENCE OF 3(3,4-DICHLOROPHENYL)-1,1-DIMETHYLUREA

EFFECTS OF HYDROXYLAMINE

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SUMMARY

Flash induced variations of the fluorescence yield have been studied at 2 °C over a long time range (at 1 μs and from 20 μs to 3 min) in chloroplasts in the presence of 3(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) with or without addition of hydroxylamine.

1. In the presence of DCMU, a polyphasic rise is observed. A first fast rise (<1 μs) is ascribed to the transfer of the positive charge from the primary Photosystem II donor Chl to a secondary donor Y. Two hypotheses are proposed to interpret the existence of the subsequent slower increase ($t_{\frac{1}{2}} \cong 70$ μs) which then follows the initial fast rise.

2. The effects or various hydroxylamine concentrations have been studied with a sufficient incubation time to inactivate the secondary donors Y and Z. This inactivation leads to a complete inhibition of the ability to emit oxygen. Under these conditions, the initial fast rise (<1 μs) is inhibited as shown by den Haan, G. A., Duysens, L. N. M. and Egberts, D. J. N. (1974) Biochim. Biophs. Acta 368, 409–421, and the oxidized Chl^+ is reduced by an auxiliary donor D. The slow fluorescence rise observed after destruction of Y and Z has a similar kinetic behaviour to that observed in the presence of DCMU only and is polyphasic. In the presence of 10 mM hydroxylamine, the constant rate of the back reaction k_1 between Chl^+ and the primary acceptor Q^- is estimated to be approx. $(135\ \mu s)^{-1}$ while the transfer of the positive charge from Chl^+ to D has a rate constant k_2 of approx. $(105\ \mu s)^{-1}$.

3. In the presence of hydroxylamine concentrations higher than 10 mM, there appears a rise in the 1–20 μs range ascribed to a direct reduction of oxidized Chl^+ by hydroxylamine.

4. In chloroplasts treated with 10 mM hydroxylamine for 15 min and washed afterwards, the rate constant k_3 of the back reaction between D^+ and Q^- is estimated to be approx. $(100\ ms)^{-1}$ which leads to a value of about 700 for the equilibrium constant between Chl and D. Hydroxylamine added under these conditions is able

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Abbreviation: DCMU, 3(3,4-dichlorophenyl)-1,1-dimethylurea.

to reduce D^+. The rate constant k_4 of this reduction is estimated to be $(350\ ms)^{-1}$ in 0.1 mM hydroxylamine.

INTRODUCTION

The model generally proposed for System II centers includes a primary electron donor, the photoactive chlorophyll a_{II} Chl and a primary electron acceptor Q. Duysens and Sweers [1] showed that Q quenches System II fluorescence in its oxidized but not in its reduced form. More recently, Okayama and Butler [2] and Butler et al. [3] have shown that the primary donor Chl is also a quencher in its oxidized form Chl^+. After the primary charge separation, the positive charge on Chl^+ is successively transferred to a series of donors whose number is at present unknown. The oxidizing equivalent is stabilized on the donor Z which is the final charge accumulating complex involved in the water-splitting process.

Following a brief saturating flash given to chloroplasts, the variations of the fluorescence yield reflect several phenomena: (a) whether the System II centers are active (Q oxidized) or inactive (Q reduced), the formation of a light-induced quencher T has been reported upon excitation by a high energy flash. This quencher T, first described by Duysens et al. [4] and studied by several authors [5, 6] is ascribed to a carotenoid triplet state which fully relaxes in about 15 μs; (b) the variations of the fluorescence yield monitored by the state of System II centers present an initial rapid increase in less than 1 μs [7] (Mauzerall's data suggest that the $t_{\frac{1}{2}}$ of this rise is about 30 ns [8]) which is followed by a slower increase [5, 9]. These kinetics are attributed to the reduction of oxidized Chl^+ by the secondary donors. In the absence of DCMU, the amplitude of the slow increase is markedly diminished by the subsequent fast decrease in the fluorescence yield which is due to the reoxidation of Q^- by plastoquinone. When the rate of this reoxidation is decreased by lowering the temperature to -50 °C, we have shown that this slow phase is totally insensitive to DCMU [9]. It is thus important to note that the DCMU present in all the experiments reported here is needed only to slow down the reoxidation of Q^-.

In order to avoid the perturbations due to the formation and the relaxation of the quencher T, most of the experiments described in this paper are performed after 20 μs following the actinic flash.

Hydroxylamine is known to affect Photosystem II in different ways. According to Cheniae and Martin [10], the inhibition of oxygen evolution by hydroxylamine results from the release of bound manganese attached to System II centers, thus inactivating the donors involved in the oxygen formation process. Using fluorescence techniques and hydroxylamine treated chloroplasts, den Haan et al. [7, 11] propose that Chl^+ is reduced ($t_{\frac{1}{2}} \simeq 25\ \mu s$) by an auxiliary donor D which replaces the secondary donors inactivated by hydroxylamine.

In this paper, we describe the variations of the fluorescence yield from 20 μs to several minutes following a saturating flash in chloroplasts blocked by DCMU and exposed to various concentrations of hydroxylamine.

MATERIALS AND METHODS

Fluorescence experiments were performed using a technique previously

described [9, 12, 13]. Chloroplasts were illuminated by a brief saturating Xenon flash (Verre et Quartz Co., model VQ X CAD 22, 1. 3J, 2 μs at half height or General Radio Stroboslave, type 1539A, 2 μs at half height). The fluorescence yield was measured in two different time ranges: (1) the fluorescence level was sampled at 1 μs in an experiment where the actinic flash itself was used to detect the fluorescence yield; (2) from 20 μs on following the actinic flash, the fluorescence yield was sampled by a weak detecting flash given at various dark times after the actinic flash.

Chloroplasts were isolated from market spinach, according to the method of Avron [14] and stored at -70 °C in the presence of 5 % dimethylsulfoxide. Prior to use the chloroplasts were suspended in 0.05 M phosphate buffer (pH 6.4) with 0.4 M saccharose and 0.01 M NaCl at a concentration of about 70 μg chlorophyll/ml. In all experiments, 20 μM DCMU was added before hydroxylamine. Stock hydroxylamine solutions were adjusted to pH 6.4.

Since the variations of the fluorescence yield due to the quencher T are temperature independent, all the experiments are performed at 2 °C in order to favor the slow fluorescence increase which is larger when the temperature is lowered [9].

RESULTS

According to den Haan et al. [11], the incubation time in the presence of hydroxylamine necessary to reach a total inhibition of oxygen emission is an inverse function of the hydroxylamine concentration. The effects on the fluorescence kinetics occur in the same time range; the final state reached for each concentration is identical and corresponds to the destruction of the secondary donors.

Fig. 1 shows the fluorescence yield before and at various dark times after the

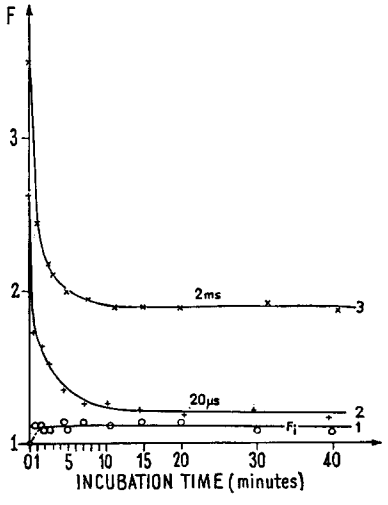

Fig. 1. Chlorophyll *a* fluorescence levels in spinach chloroplasts in the presence of 20 μM DCMU as a function of incubation time in 10 mM hydroxylamine. Curve 1: initial fluorescence level (F_i) measured on dark adapted sample before the actinic flash. This level is equal to 1 for the control (time zero of incubation). Curves 2 and 3: fluorescence levels measured 20 μs and 2 ms, respectively after the actinic flash.

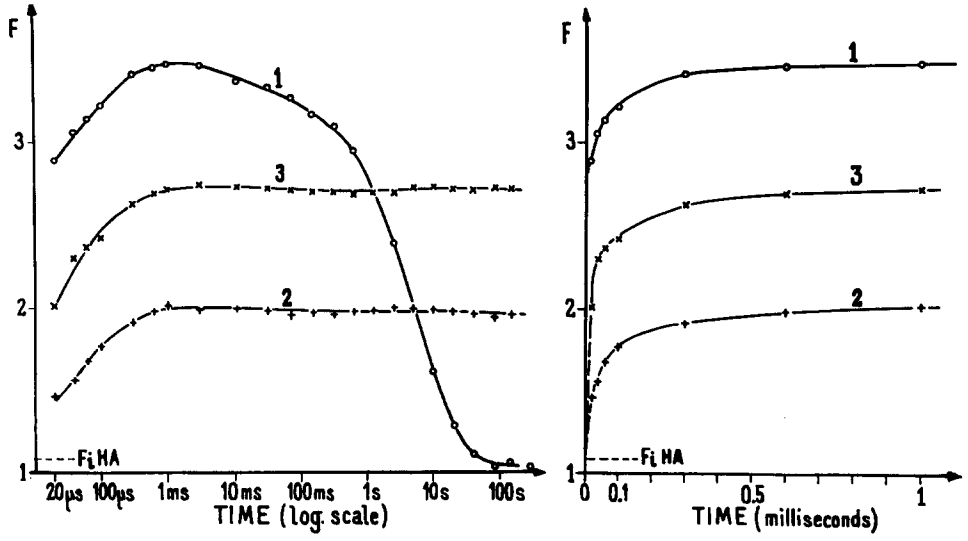

Fig. 2. Chlorophyll *a* fluorescence yield in the presence of 20 μM DCMU as a function of dark time following a brief saturating flash. Time scale is logarithmic. Curve 1, control; curve 2, incubated with 10 mM hydroxylamine for 20 min. Curve 3, with 100 mM hydroxylamine for 20 min. $F = 1$ corresponds to the initial fluorescence level measured in the presence of DCMU before the actinic flash. The dashed line F_iHA is the same measurement after addition of hydroxylamine.

Fig. 3. Same as Fig. 2, but with a linear time scale in the 0–1 ms time range.

actinic flash as a function of the incubation time of the chloroplasts in the presence of 20 μM DCMU and 10 mM hydroxylamine: for incubation times longer than 10 min, there is no significant change in the variable fluorescence parameters. The experiments presented in this paper (except the one depicted in Fig. 5) were performed in the presence of 10 mM hydroxylamine with an incubation time such that oxygen emission is totally suppressed and the variable fluorescence parameters have reached a stationary level. In the presence of 100 mM hydroxylamine, and for an incubation time longer than $\simeq$ 4 min, the fluorescence parameters behave in a different manner; this new phenomenum will be discussed later.

In Fig. 2, is depicted the variation of the fluorescence yield from 20 μs to several minutes following a saturating actinic flash. Following a short saturating flash in the presence of DCMU (Fig. 1, curve 1), there appears a rapid increase followed by a slower one. This slow increase first observed by Zankel [5] has been described in detail as a function of temperature in a previous publication [9]: at +2 °C, this rise (Fig. 3, curve 1) has a half time of approximately 70 μs but is generally biphasic and is completed in the 1–3 ms time range.

At times longer than 1 ms, the fluorescence yield decreases to the initial level and reflects the reoxidation of Q^-. This decay (Fig. 4) which is far from an exponential function, shows at least two phases: a rapid one completed in 0.3 s, followed by a slower one relaxing fully in up to 1 min.

Incubation with 10 mM hydroxylamine for 15 min induces several effects (Figs. 2 and 3, curve 2): the amplitude of the first fast increase is strongly diminished and extrapolates to a value close to the initial level F_i (as shown by den Haan et al.

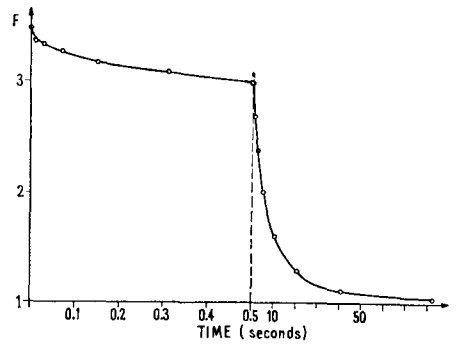

Fig. 4. Chlorophyll *a* fluorescence decay in the presence of 20 μM DCMU in the 1 ms–80 s time range (from Fig. 2, curve 1, with linear time scale).

[7] while the subsequent slow increase appears clearly polyphasic with an overall $t_{\frac{1}{2}} \simeq 50$ μs. Furthermore, the decrease beyond 1 ms has totally disappeared [15, 16]. The addition of a larger amount of hydroxylamine (up to 100 mM, Figs. 2 and 3, curves 3) does not drastically affect kinetics at time longer than 20 μs but greatly increases the level at 20 μs. Another common feature of the effect of 10 mM and 100 mM hydroxylamine is a $\simeq$ 10 % increase of the initial fluorescence level F_i measured before the actinic flash, as already mentioned by den Haan et al. [7].

Although no measurement has been performed at times shorter than 20 μs

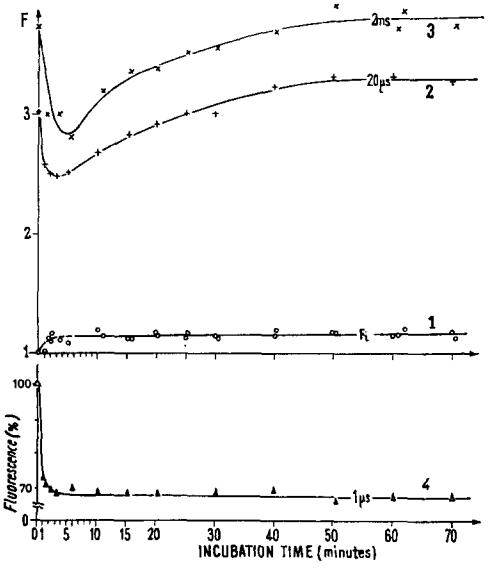

Fig. 5. Chlorophyll *a* fluorescence levels in the presence of 20 μM DCMU as a function of incubation time in 100 mM hydroxylamine. Curve 1: initial fluorescence level (F_i) measured on dark adapted sample before the actinic flash; this level is equal to 1 for the control (time zero of incubation). Curves 2 and 3: fluorescence levels measured 20 μs and 2 ms, respectively after the actinic flash. Curve 4: from a separate experiment, fluorescence level (in arbitrary units) reached during the first μs of the flash. The ordinate cannot be compared to that for curves 1–3. The same final level is reached when chloroplasts are incubated with 10 mM hydroxylamine for times longer than 10 min.

following the actinic flash, an estimation of the fluorescence level reached in the first μs was obtained using the actinic flash itself to detect the fluorescence yield. The measurement was made at a time such that a large fraction of quencher Q is already reduced to Q^- with very little formation of the light-induced quencher T.

In agreement with the data of den Haan et al. [7], our results show that the fluorescence level during the first 1 μs in the presence of DCMU is greatly decreased by the addition of hydroxylamine. These authors observed that in the presence of 1 mM or 10 mM hydroxylamine the fluorescence rise from the initial level is limited during the flash by a dark reaction. We have observed that the fluorescence level measured at 1 μs is the same whether the concentration of hydroxylamine is 10 mM or 100 mM. On the contrary, the level measured at 20 μs is higher in the presence of 100 mM hydroxylamine than in the presence of 10 mM hydroxylamine: thus, the increase of hydroxylamine concentration from 10 mM to 100 mM induces a specific increase of the amplitude of the 1–20 μs phase, whose $t_{\frac{1}{2}}$ can be estimated to be $\simeq$ 5–10 μs (Fig. 3, curve 3).

Fig. 5 shows the fluorescence yield before and at various dark times after the actinic flash as a function of the incubation time of chloroplasts in the presence of 20 μM DCMU and 100 mM hydroxylamine. While the 10 % increase of the initial level F_i and the inhibition of the fast rise measured at 1 μs are achieved in about 5 min, a continuous and parallel increase of the levels measured at 20 μs and 2 ms takes place over 50–60 min. For incubation times longer than 60 min, photoreaction II appears to be as efficient as the control (time zero of incubation) while no correlated

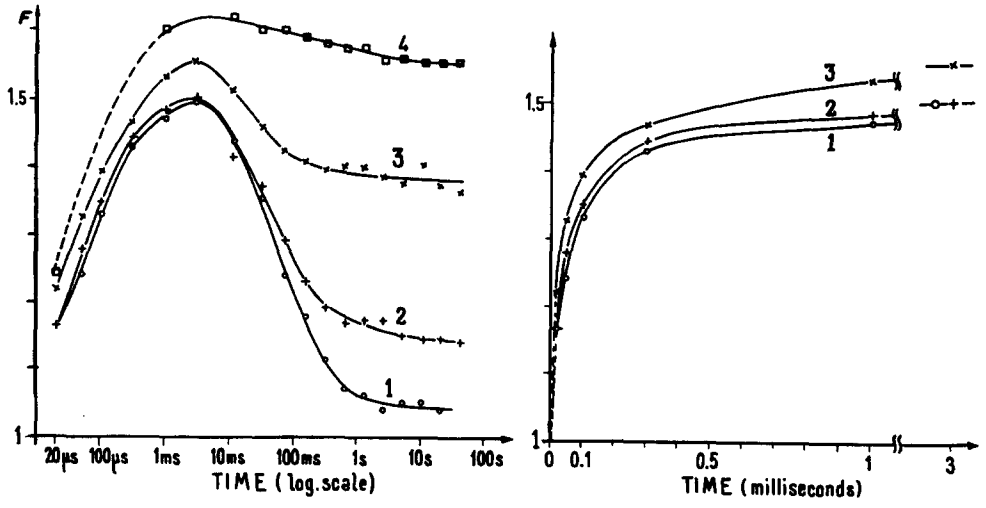

Fig. 6. Chlorophyll *a* fluorescence yield as a function of dark time (logarithmic scale), following a brief saturating flash, in chloroplasts pretreated with 10 mM hydroxylamine for 15 minutes and washed to remove hydroxylamine. $F = 1$ is the initial fluorescence yield measured on the sample before the actinic flash. Curve 1: 20 μM DCMU; curve 2: DCMU plus 0.1 mM hydroxylamine (10 min incubation); curve 3: DCMU+1 mM hydroxylamine (10 min incubation); curve 4: DCMU+10 mM hydroxylamine (10 min incubation).

Fig. 7. Same as Fig. 6, but with a linear time scale in the 0–1 ms range.

increase occurs for the first μs of the actinic flash. The 1–20 μs rise under these conditions represents about 80 % of the variable fluorescence, that is to say about 90 % of the system II centers [17]. We note that the relative amplitudes of the 1–20 μs rise and the slow one can vary from one chloroplast preparation to another.

In order to study the effects of lower concentrations of hydroxylamine, we pretreated chloroplasts with 10 mM hydroxylamine for 15 min and then washed the hydroxylamine by centrifugation and resuspension in an hydroxylamine free buffer. The variation of the fluorescence yield from 20 μs following the actinic flash for these chloroplasts is depicted in Fig. 6 (curve 1). In the 20 μs–3 ms time range, a slow markedly biphasic increase appears which extrapolates to the initial fluorescence level measured on dark adapted chloroplasts, very similar to the one observed in the presence of 10 mM hydroxylamine. On the other hand, beyond 3 ms a dramatic decrease takes place and the fluorescence yield comes back to a value close to the initial level about 50-times faster than in the control ($t_{\frac{1}{2}} \simeq 60$ ms compared to 3 s, Fig. 4). Upon addition of 0.1 mM (Figs. 6 and 7, curve 2) or 1 mM (curve 3) hydroxylamine however, no striking effect is seen on the increasing phase, but the stationary level reached at the end of the decreasing phase becomes higher as the concentration increases. Upon the addition of 10 mM hydroxylamine, we observed (Fig. 6, curve 4) a fluorescence evolution similar to the one obtained when 10 mM hydroxylamine is directly added to chloroplasts (cf. Figs. 2 and 3, curve 2). One must note however, that the amplitude of the variable fluorescence is larger in unwashed chloroplasts. The washing procedure irreversibly damages a fraction of the centers ($\simeq$ 38 %).

DISCUSSION

The existence of a submicrosecond rise in the fluorescence yield has been interpreted as corresponding to the reduction of oxidized Chl^+ by a secondary donor. As shown by den Haan et al. [7] this fast transfer is inhibited by hydroxylamine. We observed a slow fluorescence increase beyond 20 μs in the presence of DCMU as well as in the presence of DCMU plus hydroxylamine. Expressed in terms of the number of System II centers, the amplitude of this slow increase is larger in the presence of DCMU plus hydroxylamine, than in the presence of DCMU only, nevertheless the kinetics are similar. Either this similarity is a coincidence or indicates that the same rate limiting reaction is functioning both in the presence and absence of hydroxylamine. We discuss below two models which consider this problem as well accounting for the biphasicity of the fluorescence rise.

A first hypothesis previously proposed [9] is: the fast rise observed in the presence of DCMU results from a rapid equilibration (< 1 μs) (low equilibrium constant) between Chl^+ and Y. The slow increase would then represent the slower reduction of Y^+ by a subsequent donor. The polyphasicity of this increase would thus imply the existence of several redox species between Y and the charge accumulating complex Z involved in oxygen formation (Fig. 8, Scheme 1).

In this hypothesis, the effect of hydroxylamine would be as stated by den Haan et al. [7] to inactivate the secondary donor Y. The oxidized Chl^+ would then be reduced by an auxiliary donor D, the charge transfer between Chl and D being much slower than between Chl and Y. In these conditions, the kinetic similarity of the slow increase in the presence or in the absence of hydroxylamine would be a coincidence.

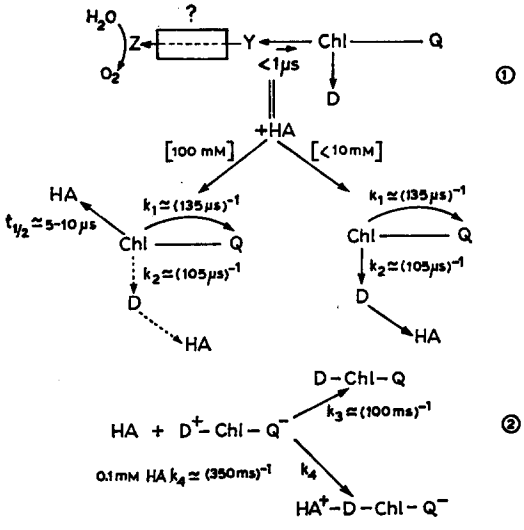

Fig. 8. Schematic representation of the effect of hydroxylamine on System II centers in the presence of DCMU. Chl, photoactive chlorophyll a_{II}, primary donor; Q, primary acceptor; Y, secondary donor; Z, charge accumulating complex, involved in water-splitting mechanism; D, auxiliary donor; HA, hydroxylamine. The arrows indicate the direction of movement of the positive charges. The kinetics shown are those obtained at 2 °C, and for sufficient incubation time with hydroxylamine to totally destruct the secondary donors Y and Z. Scheme 1: in the presence of 100 mM hydroxylamine, in most of the centers Chl^+ is directly reduced by hydroxylamine (solid arrow, $t_{\frac{1}{2}} \simeq 5$–10 μs estimated from Fig. 3, curve 3). For the other centers, where Chl is not directly accessible to hydroxylamine, electron donation from hydroxylamine occurs via D, (dashed arrows). Scheme 2: reduction of D^+ in DCMU+hydroxylamine treated chloroplasts. See Text.

Another way to interpret these results might be a heterogeneity among the centers involved in the DCMU fluorescence rise. The centers responsible for the fast rise would have their photoactive chlorophyll linked to the "fast" donor Y, while in those responsible for the slow increase, the chlorophyll would receive an electron only from donor D. The effect of hydroxylamine would be to inhibit the Chl → Y reaction, leading to the reduction of Chl^+ by donor D only. This hypothesis would explain the close values found for the half-time of the slow rise with or without hydroxylamine. The two types of centers could be similar to those proposed by Melis and Homan [18, 19].

It is important to note that under the same experimental conditions similar biphasicity is observed in *Chlorella*: thus, it is unlikely that the slow increase shown by chloroplasts would be due to the inactivation of a fraction of the centers during the preparation process, e.g. by a release of bound manganese.

Although den Haan et al. [20] propose that Y and D are different, one can imagine that it is actually the same donor site which could be modified chemically and/or structurally by hydroxylamine. An argument which favors this hypothesis comes from EPR measurements where two signals SII_{vf} and SII_f. are observed in intact and Tris washed chloroplasts, respectively. These two signals have a similar EPR spectrum and a similar amplitude.

Nevertheless, in the absence of clear cut characterization of the different donors we shall assume in the rest of the discussion that Y and D are different.

From curve 2, Fig. 2, it appears that the final fluorescence level reached in the presence of DCMU plus 10 mM hydroxylamine is $\simeq$ 36 % of the maximum level of the control (curve 1). Taking into account the non-linear relation between the fluorescence yield and the concentration of System II centers [17], this means that only $\simeq$ 56 % of the centers are stabilized in the D^+–Chl–Q^- or HA^+–D–Chl–Q^- form, due to the competition between the back reaction $Chl^+–Q^- \xrightarrow{k_1}$ Chl–Q and the charge stabilization $D–Chl^+ \xrightarrow{k_2} D^+–Chl$. We have found that this competition varies slightly from one chloroplast preparation to another. A rough estimation of the rate constants k_1 and k_2 can be obtained assuming an exponential increase with an overall time constant of $\simeq$ 60 μs: $k_1+k_2 = 1/60$ and k_2/k_1: 0.56/0.44. Calculation of the rate constant k_1 for the back reaction gives $k_1 \simeq (135\ \mu s)^{-1}$, which is not far from the values estimated by Lavorel [21] and Haveman and Mathis [22]; the rate constant k_2 for the charge stabilization is $k_2 \simeq (105\ \mu s)^{-1}$. These values are only approximate, as the fluorescence increase is obviously non exponential.

In the presence of 100 mM hydroxylamine, the appearance of a greater rise in the 1–20 μs range suggests that for a fraction of the centers, the oxidized Chl^+ is reducible directly by hydroxylamine. The slow increase is due to those centers where Chl^+ still reacts with D. The fraction of the centers directly accessible to hydroxylamine increases with the incubation time.

Chloroplasts, treated with hydroxylamine and then washed, show a rapid back reaction. Assuming it is exponential with a time constant of $\simeq (100\ ms)^{-1}$, this reaction appears to be 500–1000 times slower than the back reaction $Chl^+Q– \xrightarrow{k_1}$ Chl–Q in which k_1 has been estimated to be $(135\ \mu s)^{-1}$. This suggests that the equilibrium constant between Chl and D is of the order of 700, corresponding to a difference in potential between Chl/Chl^+ and D/D^+ of about 170 mV. (i.e. if Chl/$Chl^+ \simeq +0.8$ V, D/D^+ would have a potential of $\simeq +0.630$ V).

Upon addition of 0.1 mM or 1 mM hydroxylamine, the final level reached is increased, pointing out a reduction of D^+ by hydroxylamine as shown by den Haan et al. [7, 11]. The fraction of the centers stabilized in the Q^- form is determined by the competition between the back reaction $D^+–Chl–Q^- \xrightarrow{k_3} D–Chl–Q$ and the rate of the reduction of D^+ by hydroxylamine $HA+D^+–Chl–Q^- \xrightarrow{k_4} HA^+–D–Chl–Q^-$ (Fig. 8, Scheme 2).

In the presence of 0.1 mM hydroxylamine (Fig. 6, curve 2), about 22 % of the centers have stabilized the positive charge on hydroxylamine; from $k_4/k_3+k_4 = 0.22$ and if we accept (Fig. 6, curve 1) that k_3 is $\simeq (100\ ms)^{-1}$, the rate constant k_4 is $\simeq (350\ ms)^{-1}$. This value is markedly different from the one measured in *Chlorella* by den Haan et al. (cf. ref. 11, k_4 1.1 · s^{-1}, 0.5 mM hydroxylamine, 24 °). However, in addition to the difference in the material, one must note that the den Haan et al. measurements are performed in repetitive flash experiments while our measurement is performed after a single flash illumination.) The rate of this reaction increases with hydroxylamine concentration with a non linear relation; at 100 mM, a decreasing phase is no more observed, the reduction of D^+ by hydroxylamine being much faster than the back reaction.

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